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Coaggregation in a microbial predator-prey system affects competition and trophic transfer efficiency

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ABSTRACT

Emerging interactions are key determinants of system productivity and efficiency in plant and animal communities, whereas their importance for planktonic microbial assemblages is unknown. We studied the relationship between two aquatic bacterial strains and a protistan predator with respect to cooperation and system efficiency. While competitive exclusion of one bacterial strain was observed in grazer-free co-cultures, the presence of the predator induced the formation of large co-aggregates and allowed a coexistence of both strains. The aggregates moreover provided a substrate for attachment of the predators. The interactions in the more complex community, i.e., microbial flocks composed by both bacterial strains and predators, led to a significantly increased transfer efficiency of dissolved organic matter to the higher trophic level than in systems with two species only. Similar emerging interactions might also play a role in complex microbial assemblages in natural aquatic systems, e.g., on suspended organic aggregates.

KEYWORDS: microbial coaggregation, prey-predator interactions, cooperation, system efficiency, Brevundimonas, Arthrobacter agilis, Poterioochromonas, community ecology

INTRODUCTION

The integration of community ecology with ecosystem science has resulted in a drastic increment of the variables that need to be considered for an analysis of natural systems. Our ability to understand the behavior of such systems from the known interactions between organisms is still limited, also because many important interactions may, in fact, be unknown. Simplified artificial systems allow the precise definition and quantification of an arbitrary number of ecological variables and may thus help to reveal such indirect (usually hidden) interactions. Microbes are ideally suited models for laboratory-based systems, since they quickly form large populations and their growth conditions can be precisely controlled (Bohannan and Lenski 2000). Experimental microbial communities not only shed light on interactions within the microbial world (Jessup et al. 2005), but can also provide clean tests of ecological theories (Drake et al. 1996) to bridge the gap between theory and field ecology (Lawton 1995).

Laboratory microcosms have been fundamental for gaining a better understanding of the role of predation and competition in shaping microbial communities. Experimental studies have revealed the ability of bacteria to escape from protozoan predation by various means, e. g. the release of toxic substances (comprehensive review by Jürgens and Matz 2002), high cell motility (Matz and Jürgens 2005), or by developing large filamentous morphotypes (Shikano et al. 1990), and some of these responses may even be triggered by infochemicals (Corno and Jürgens 2006, Blom et al. 2010b). The formation of cell clusters, ranging from microcolonies composed by a few cells to large aggregates often stacked within self-excreted exopolymers, has also been proposed as an effective anti-predation mechanism (Jürgens and Güde 1994, Hahn et al. 2004). At the same time, the multispecies microbial consortia aggregated on organic debris (e.g., senescent algal cells) and larger organisms (e.g. zooplankton) have been identified as hot spots of high production in oceans (marine snow, Alldredge and Silver 1988) and lakes (Grossart and Simon 1993) and they represent ideal habitats for enhanced

microbial substrate uptake and dispersal (Grossart et al. 2010). The simultaneous action of top-down and bottom-up factors on microbial (co-)aggregates in the pelagic zone suggests that such associations might represent well-suited systems to explore the impact of hidden ecological interactions between the involved predator and prey species in a spatially limited environment.

So far, most studies have focused on determining the relative impact of predation and competition on microbial genotypic diversity or phenotypic distribution only (Matz et al. 2004, Corno and Jürgens 2008). This experimental design tends to exclude other possible ecological interactions that would result in undesired complications of the system. Specifically, the effects of mutualism or neutralism have been circumvented by the artificial selection of species, and the possible importance of novel, emerging interactions (e.g., the recycling of nutrients excreted by predators during grazing and made available to the prey) has been discussed only (Goldman and Caron 1985, Nagata and Kirchman 1992).

In this study we demonstrate the increase of system complexity when an emerging interaction is introduced in a simple three species system consisting of two bacterial strains and a protistan predator. The two bacterial were able to coexist only if predation pressure reduced the effect of competitive exclusion. Moreover, a neutralistic interaction between the strains (coaggregation) substantially raised the overall biomass production efficiency of the system, resulting in a direct advantage for the predators. Our observations highlight the potential importance of emerging interactions between coaggregating microbes and their predators for explaining the high levels of productivity of suspended microbial aggregates in pelagic habitats.

MATERIALS AND METHODS

Isolation of model organisms

Two bacterial strains from different phylogenetic linages were isolated from an enrichment culture from Lake Zurich in autumn 2009. A lake water inoculum was first filtered through 0.8 µm and then 1:10 diluted with artificial lake water medium (ALW) (Zotina et al. 2003) amended with peptone, yeast extract and glucose (final concentration, 3 mg L^{-1} each). The Dissolved Organic Carbon (DOC) concentration in ALW medium was determined with a Shimadzu TOC-5000 after filtration of the samples through GF/F (Whatman) precombusted glass fiber filters.

Predation pressure in enrichments was established by addition of an axenic bacterivorous nanoflagellate, *Poterioochromonas sp.* strain DS (final concentration, about 1:1000 of bacterial abundances), in order to promote the development of bacteria with antipredator defense strategies. Being common in freshwaters, easy to cultivate, able to attach to substrates, and voracious interception-feeders, many members of the family *Ochromonadaceae* (e.g. *Poterioochromonas*) can be considered well-suited model organisms to study the impact of predation by mixotrophic flagellates on bacteria (Rothhaupt 1997). A *Poterioochromonas* sp. was found to release a complex mixture of secondary metabolites during grazing (Blom and Pernthaler 2010), including chemical cues that affect bacterial growth and morphology (Corno and Jürgens 2006), and this predator moreover had a defined preference for medium sized bacterial cells or clusters (Pernthaler et al. 2001, Corno and Jürgens 2008).

After 120 h of incubation in the dark, life subsamples were stained with the nucleic acid dye 4′,6-Diamidino-2-phenylindol (DAPI, Sigma-Aldrich, final concentration 1µg ml^{-1}) and analyzed by flow cytometry in order to detect cell aggregates according to their DNA content and light scatter properties. Aggregates with a diameter between 20 µm and 70 µm were then sorted on a solid growth medium (ALW-medium Agar, 2.2.1.3) in Petri dishes (70 aggregates $dish^{-1}$). After several days of incubation, colonies were selected that visibly consisted of more than one strain and the participating strains were isolated. One of the most commonly isolated co-colony types was composed of

Arthrobacter agilis strain GC027 and *Brevundimonas* sp. strain GC044, as identified by partial sequencing of their 16Sr RNA genes (deposited in GenBank under accession numbers JN009621 and JN009622). These two strains, together with *Poterioochromonas sp.* strain DS, were used as models to investigate the interplay of foraging, competition and possibly cooperation within a simplified community of coaggregating microbes and their predators.

Experimental design

Four replicate batch cultures of each single strain and 4 cocultures of the two strains were established both, with and without predators. Microbes were inoculated into 120 ml of ALW medium at concentrations of about 1.5 x 10^6 cells ml^{-1} (pure cultures) or of 0.75 x 10^6 cells ml^{-1} from each strain (cocultures). All bacteria had been pre-cultured in ALW medium for 24-36 h prior to inoculation. Treatments with predation were additionally inoculated with about 1.5 x 10^3 cells ml^{-1} of *Poterioochromonas sp.* strain DS (i.e., final concentrations of approximately 1:1000 to bacterial cells). The experiments ran for 168 h at room temperature in the dark. Subsamples of 5 ml were collected every 24h for flow cytometric and microscopic analyses.

Sample preparation, counting and image analysis

Samples for flow cytometric counting were immediately fixed with ice-cold glutaraldehyde solution (Fluka, final concentration, 2.5% v/v), followed by staining with 10µl ml^{-1} of DAPI and 8-10 minutes of incubation in the dark (Kepner and Pratt 1994). Flow cytometric analyses were performed on an inFlux V-GS flow cytometer (Becton Dickinson Inc.) equipped with a UV-laser (355 nm excitation wavelength, 60mW) and a blue laser (488 nm excitation wavelength, 200mW) as light sources. The minimal number of recorded events was 200000. Bacterial populations were quantified in dot plots of 90° angle light scatter (side scatter, SSC) vs. DAPI fluorescence (460 nm, FL1) while

populations of *Poterioochromonas sp.* strain DS were analyzed in plots of SSC vs. autofluorescence (530 nm, FL2). The bacterial growth types were operationally defined, based on prior sorting and microscopic inspection, as (i) single cells: events located between 2 and 50 SSC units and lower than 100 FL1 units; (ii) aggregates: all events with higher SSC and higher FL1 signal than that of *Poterioochromonas sp.* strain DS cells; and (iii) microcolonies: all events located between the gates defining aggregates and single cells.

Fluorescence *in situ* hybridization and catalyzed reporter deposition (CARD-FISH) was performed on subsamples of cells filtered on membrane filters (type GTTP, Millipore (Sekar et al. 2003)). The probes HGC69a (Roller et al. 1994) and ALF968 (Glöckner et al. 1998) were used for the identification of *A. agilis (Actinobacteria*) and *Brevundimonas* sp. (*α-proteobacteria*), respectively. Preparations were counterstained with DAPI and analyzed by epifluorescence microscopy (Axio Imager M1, Zeiss) to determine the proportions of hybridized cells, for size classification of the aggregates, and to estimate the respective proportions of the two strains in coaggregates. At least 1000 cells (and 250 aggregates) were analyzed per replicate. Images for the sizing of aggregates and single cells were captured with a Cool-1300Q camera (Vosskühler GmbH) and evaluated by image analysis (LUCIA D, Laboratory Imaging SRO). Aggregate size was approximated by determining the maximal Feret dimension (Fe_{max}) of each aggregate detected on CARD-FISH stained filters, and aggregates were grouped into size classes of 10 µm. Cell clusters with Fe_{max} <10 µm were considered as microcolonies. The volumes and organic carbon content of bacterial and flagellate cells was estimated according to Loferer-Krössbacher et al. (1998) and Menden-Deuer et al. (2001).

Approximations of total cell numbers, statistical analyses

The average number of cells per aggregate/microcolony was separately estimated by the median SSC values of microcolonies and aggregates divided by the median SSC value of single cells in each

cytogram of the flow cytometric analysis. The dominance of either bacterial strain in coaggregates was assessed by epifluorescence microscopy of CARD-FISH stained samples. Since a precise quantification of this qualitative result was not feasible and coaggregates typically contained only a small proportion of cells from the non-dominating strain, the dominating strain was conservatively assigned a relative proportion of 95% of total cells in the aggregate.

The relative impact of competition vs. predation on the bacterial populations was estimated by relating the number of edible prey (i.e. single cells) to the number of predators at 96 h (Fenchel 1986). Ratios of predators to edible prey lower than 1 : 1000 were considered as indicators for control of bacteria by predation, while higher values suggested competition for resources as the main limiting factor. We also estimated the amount of bacterial carbon that would be necessary to explain the measured flagellate biomass keeping the conservative value given by Fenchel (1982) on the amount of bacterial carbon actually utilized by *Ochromonas* sp. after ingestion (43% of the total), and thus actually transferred to the upper trophic level (ingested prey biomass = estimated predator biomass x 0.43).

Treatment series were first tested for normal distribution and then for significant differences by repeated measures analysis of variance (rm ANOVA): changes in growth type distribution against time, were tested for single strains and communities during the stationary growth phase (96-168h); significant differences in overall and in specific growth types abundance between treatments (96-168h) (pure *vs* co-cultures and no predation *vs* predation) were also tested for each strain. Single time points were compared by one-way ANOVA (again after testing for normality), with comparison of the means by Tukey's post hoc test. All analyses were carried out using SigmaPlot, version 11 (Systat Software Inc.).

RESULTS

A comparison of the overall abundances of each strain in pure and cocultures and in treatments with or without predators during the stationary growth phase (96-168h) showed a significantly (2-way rm ANOVA, $P < 0.001$ Table 1) lower number of *A. agilis* in cocultures independent of the presence of the grazers, while predation did not have any significant impact on *A. agilis* numbers. A similar (but not significant) trend could be detected for *Brevundimonas* sp. ($P = 0.051$). In cocultures without predators *Brevundimonas* sp. reached smaller numbers compared to the ones in pure culture ($P < 0.001$) while under predation it reached significantly higher abundances ($P = 0.024$Table 1). Grazing had always a significant impact on the overall abundance of *Brevundimonas* sp. ($P < 0.001$).

A. agilis strain GC027 reached total numbers of 4.4×10^6 cells ml^{-1} when grown in pure cultures without predation, and of 5.3×10^6 cells ml^{-1} when exposed to grazing by *Poterioochromonas sp.* strain DS, without any significant impact of predation on the total numbers of formed cells (Fig. 1). Conversely, *Brevundimonas* sp. grew to 31.9×10^6 cells ml^{-1} without predators, but was significantly reduced under predation. The coculture of the two strains reached significantly higher numbers without than with predators (Fig. 1, first row). Both, *A. agilis* and especially *Brevundimonas* sp. produced microcolonies of 3-15 cells, but to a different extent in the various treatments (Fig. 1, second row): *A. agilis* featured a rather constant small number of microcolonies (harboring between $0.4\text{-}0.6 \times 10^6$ bact ml^{-1}), independent of the presence of the predators. *Brevundimonas* sp. developed tenfold more microcolonies under predation than in the grazer-free treatments. In both treatments, the number of microcolonies grew only during the exponential growth phase (first 72 hours), while they were stable during the stationary growth phase ($P > 0.05$, Table 1). In the coculture, microcolonies were far more abundant and contained about 40 times more cells in the presence than in the absence of predators. Moreover their numbers significantly increased also during the stationary phase ($P < 0.01$), with a maximum after 144 hours. In pure culture *A. agilis* formed relatively small numbers of larger

aggregates (harboring about 0.6 x 10^6 bact ml^{-1}) that were similar in treatments with and without predators, whereas *Brevundimonas* sp. did not form aggregates in either treatment (Fig. 1, third row).

High numbers of aggregates were formed only in those cocultures that also featured flagellate predation, and aggregates moreover significantly increased during the stationary growth phase ($P < 0.001$), both in absolute and relative abundances. By contrast, both the proportions and numbers of single cells continuously declined in this treatment during the stationary growth phase ($P < 0.01$, Table 1).

The relative distribution of the three growth types (single cells, microcolonies, and aggregates, Fig. 2A) during the stationary growth phase thus revealed no substantial differences between treatments with and without predators for *A. agilis*. *Brevundimonas* sp. in pure cultures without predators, as well as the ungrazed coculture were dominated by single cells, with proportions >98% of total cells number. Under predation pressure, *Brevundimonas* sp. formed a substantial proportion of microcolonies (44% and 48%) in pure cultures and cocultures, while almost one quarter of bacteria were present in aggregates (24%) only in the coculture. CARD-FISH analysis revealed that microcolonies predominantly consisted of a single strain, while the majority of aggregates contained two bacterial strains (Fig. 2B). The proportion of coaggregates increased with aggregate size, and all aggregates >60 µm were composed of both species. Coaggregates dominated by *Brevundimonas* sp. represented >90% of the total number of all coaggregates in all samples. Usually the number of *A. agilis* cells did not exceeded 10% of the total cell numbers in a coaggregate (mean, 5%).

A reduction in the total numbers of *A. agilis* compared to stationary phase pure cultures was observed in co-cultures independent of predation (Table 1; Fig. 3, upper bar chart), By contrast, the distribution of cell types was predation-dependent. Specifically, there was a drastically lower number of single cells that almost disappeared in the grazed coculture. Conversely, the abundances of microcolonies and aggregates were comparable in pure cultures and in the cocultures with predators,

but were substantially lower in the grazing free cocultures. Thus, the total cell numbers of (aggregated and free-living) *A. agilis* were comparable in stationary phase cocultures with and without predators . However, it was increasingly outcompeted in treatments without predators, being reduced to a proportion of only 2% of cells at the end of the experiment (compared to 8% at 96 hours). By contrast, its relative proportions in the grazing- exposed cocultures rose from 6 to 10% during the same period (Fig. 3, upper line and scatter plot). In this treatment the total numbers of *A. agilis* also increased slightly but significantly ($P = 0.027$) during the stationary growth phase, resulting in a community of about 1×10^6 cells ml^{-1} at the last time point.

Cell density of *Brevundimonas* sp. during stationary growth phase in cocultures was lower than in pure cultures without predation, whereas the opposite was the case for the grazing-exposed variants (Fig. 3, lower bar graph). Moreover, also the distribution of *Brevundimonas* sp. within the various growth types was highly affected by predation, with a large number of cells present in microcolonies or in aggregates under grazing pressure. There was a clear tendency of *Brevundimonas* sp. to drive *A. agilis* towards extinction in coculture without predators (Fig. 3, upper line and scatter plot), while no such trend was observed under predation (where *Brevundimonas* sp. nevertheless still accounted for >90% of the total cells number).

Poterioochromonas sp. strain DS feeding on *A. agilis* only grew to 3.1×10^3 cells ml^{-1}, while it formed more than twice this abundance in the presence of *Brevundimonas* sp. (Fig. 4, line and scatter plot). However, the flagellates reached almost ten times higher densities in the cocultures of the two strains. Moreover, a significantly larger proportion of *Poterioochromonas sp.* strain DS cells in this treatment was attached to bacterial aggregates (approximately 40% vs. <5% of all flagellates, Fig. 4, pie charts). A positive relationship between aggregate size and the attachment of the predators could be established (Fig. 5), especially for coaggregates mainly composed of *Brevundimonas* sp. cells. By

contrast, the few aggregates dominated by *A. agilis* apparently did not attract the attachment of *Poterioochromonas sp.* strain DS.

A significantly higher ratio of prey cells per predator was observed in the two pure cultures than in the coculture (one-way ANOVA, $P < 0.001$; Fig. 6A). The efficiency of the systems in terms of biomass production was determined by comparing the amount of available DOC in the medium (5.261 mg C L^{-1}) with the estimated total biomass of bacteria and protists at the end of the experiments (Fig. 6B). In the absence of predation, pure cultures of *Brevundimonas* sp. appeared to be slightly more efficient in transforming DOC into biomass than the coculture, and they were four times more efficient than *A. agilis*. The presence of predators slightly increased the total biomass production (i.e., the summed biomass of bacteria and flagellates) in the *A. agilis* cultures, while reducing that of the *Brevundimonas* sp. cultures by half. The presence of predators in cocultures resulted in a three times higher biomass production efficiency compared to predator-free variants, resulting in a transformation of about 40% of DOC into biomass (Fig. 6B). Moreover, a pronounced allocation of biomass into flagellates (>90%) was observed in the grazed cocultures, whereas bacterial biomass in these treatments approximately resembled that of the grazed pure cultures. The estimate of the bacterial carbon required to form the measured flagellate biomass (Fig. 6B, white bars) suggested that about 80% of the total DOC provided by the medium was incorporated into bacteria in cocultures under predation (5 and 2.3% in *Brevundimonas* sp. and in *A. agilis* grazed pure cultures, respectively) and that about 43% of it was subsequently transferred to the predators.

DISCUSSION

Although both bacterial model species were obtained from lake water enrichments, they nevertheless differ in their ecological affinity to lacustrine habitats and probably occupy very different niches. The gram-positive *A. agilis* is a well-studied plant pathogen that can be found in freshwaters

only occasionally (Hervàs et al. 2009, Newton et al. 2011), while members of the genus *Brevundimonas* are common in freshwaters, where some adapt well to low substrate conditions (Newton et al. 2011). The two bacterial strains differed, both, in their palatability and their respective substrate uptake efficiency: *Brevundimonas* sp. had a limited capacity to escape predation, but exhibited highly efficient substrate uptake (Fig. 1). *A. agilis* was less competitive yet considerably more resistant against predation, possibly due to its gram-positive cell wall (Iriberri et al. 1994) (Fig. 1). Co-existence in predation-free cocultures led to an overall reduction of the numbers of both bacteria (compared to their pure culture performance) and eventually to the exclusion of *A. agilis* (Fig. 3). The flagellate *Poterioochromonas* sp. strain DS is a common model grazer in laboratory experiments due to its uncomplicated maintenance and cosmopolitan occurrence (Corno and Jürgens 2006, Blom et al. 2010b). It exhibits high feeding rates, e.g. between 10 and 120 cells h^{-1} at bacterial densities between 2 and 10 x 10^6 cells ml^{-1}, with increasing predation efficiency at higher prey abundance (Boenigk et al. 2002). While *Poterioochromonas* sp. strain DS is considered to be mainly a planktonic interception feeder, it has also been observed to forage when attached to surfaces (Boenigk et al. 2002). It should be noted that by using clonal cultures of the predators and prey we largely eliminated genotypic microdiversity (Jaspers and Overmann 2004), thereby possibly also reducing their phenotypic diversity (van Gremberghe et al. 2009).

A strikingly different response of the two strains to grazing was reflected by the relative abundances of growth types in grazing-free treatments vs. under predation: while *A. agilis* was almost equally distributed into single cells, microcolonies and aggregates irrespective of the presence of predators, the proportions of *Brevundimonas* sp. growth types was highly affected by predation (Figs. 2, 3). Specifically, the medium sized single cells of *Brevundimonas* sp. dominating in treatments without predators likely represented the optimal growth type to profit from bottom-up factors (Koch 1996), but also fell into the preferred prey size range of the flagellates (Pernthaler 2005). This was

suggested by a loss of >50% of total *Brevundimonas* sp. abundances under predation and the simultaneous re-allocation of a large fraction of the remaining cells into microcolonies (Fig. 1 ,2).

A predominance of coaggregates (Fig. 2) was only observed under predation, and must thus be considered as a response to flagellate grazing. Predation can affect bacterial morphology, activity, phenotype, and growth state (Corno et al. 2008, Blom and Pernthaler 2010); it might have promoted coaggregation in various ways, e.g. by providing direct or indirect chemical stimuli (Blom et al. 2010a). Most coaggregate exhibited a conspicuous distribution of bacterial cells: a small number of *A. agilis* in central positions were surrounded by groups of *Brevundimonas* sp. cells that were often arranged in microcolonies (Fig. 2, 5). Adhesion mechanisms between bacterial strains are known to be highly specific and can mediate both, ecological and physiological advantages to the coaggregating strains, such as the transfer of chemical signals, exchange of genetic information, metabolic cooperation, and protection from predation (Wimpenny and Colasanti 2004). Microbial coaggregation is generally regulated at the level of cell membranes and involves protein-saccharide interactions (Buswell et al. 1997). Due to the difference in their cell membrane composition (Kolenbrander 1989) our bacterial strains are thus theoretically well suited for coaggregation.

The formation of coaggregates in the grazed cocultures provided a partial refuge for *Brevundimonas* sp.: despite substantially higher predator densities a significantly larger proportion of cells from this strain were able to prevail in cocultures than in pure cultures by gathering in coaggregates (Figs. 3, 4). Coaggregation could in fact be considered a rather successful strategy for *Brevundimonas* sp.: it likely had no negative impact on the ability of these bacteria for substrate uptake and utilization (the defence costs paradigm, Coustau et al. 2000), as concluded from their high potential growth (Fig. 6B) under extreme predation (Figs. 4, 6A). By contrast, *A. agilis* did not seem to gain a comparable advantage from coaggregation: its abundance dropped by 3-4 fold in cocultures under predation compared to its growth in pure culture. It is thus conceivable that *A. agilis* cells were

passively “trapped” between aggregating microcolonies of *Brevundimonas* sp., possibly causing substrate limitation (shadow competition, Wilson 1974). However, a closer look revealed that the rapid competitive exclusion of *A. agilis* over time -as observed in non-grazed cocultures- appeared to be arrested or even reversed by the presence of the predators and their selective grazing on *Brevundimonas* sp. (Fig. 3, inset). Thus, despite its disadvantageous spatial disposition, *A. agilis* indirectly obtained an advantage that allowed for its persistence in the system (e.g., the high predation pressure on its competitor). This, in turn favored the presence of the large (co)aggregates, which were not formed in grazed pure cultures of *Brevundimonas* sp. (Fig. 1).

The significantly higher total bacterial abundance in the cocultures under predation than in both grazed pure cultures (rm ANOVA, $P<0.5$ vs *Brevundimonas* sp. and $P<0.01$ vs *A. agilis* pure cultures, respectively, Fig. 1) might lead to the premature conclusion that (co)aggregation more than anything represented a successful anti-grazing mechanism, as also proposed for bacterial biofilms (Rickard et al. 2002) and pure cultures of specific planktonic bacteria (Blom et al. 2010a). However, the unexpected success of the predators in the cocultures (Fig. 4) and the large proportion of *Poterioochromonas* sp. attached to the coaggregates (up to 40% of the total predator population, Fig. 5) suggest that coaggregation should not primarily be regarded as anti-predation mechanisms. Instead, the appearance of coaggregates colonized by predators (Fig. 5) has to be considered as evidence for the formation of a new habitat (Fenchel 1986) for *Poterioochromonas* sp.. This novel system feature, moreover, helps to explain why the strong reduction of free living single bacterial cells in the grazed cocultures (and thus of suitable prey for flagellates) did not have a deleterious effect on predator abundances. Thus, coaggregation represented a newly emerging system property that could not be predicted from the interactions between single prey species with each other or with the predator, and that can only be explained by simultaneous interactions between the two bacterial strains and the predators.

Limiting our observations to the prey species, the emerging coexistence would seem to have only a relatively small impact (Fig. 6), as demonstrated for other organisms sharing a multispecific group (the selfish herd effect, Hamilton 1971, Rayor and Uetz 1993). However, the biomass production of the experimental system as a whole was substantially elevated in the grazed cocultures, where more than one third of the total DOC provided by the medium was transferred to *Poterioochromonas* sp. (Fig. 6). By contrast, DOC transfer to the predator was 10 and 20 times lower in pure cultures of *Brevundimonas* sp. and *A. agilis*, respectively (Fig. 6). This is evidence that the enhanced biomass production of flagellates was not due to osmotrophic DOC uptake. Thus, the success of the flagellates in cocultures with both prey species can only have been due to a large number of edible bacterial cells. This, in turn allows estimating the amount of DOC channeled through bacterial biomass into predators (Fig. 6): about 80% of the total DOC provided to the system was transformed into biomass in the grazed cocultures, which is more than 20 times higher than the average C content of the bacterial standing stock during the stationary growth phase.

Coaggregates thus represented hot spots for bacterial production and the channeling of DOM to the next trophic level. Their number and proportions continuously rose during the experiment, while at the same time the number of free living bacterial cells declined drastically (Fig. 1). Concomitantly there was a rapid increment in the abundance of the predators until 96 h. Thereafter, total flagellate abundances stagnated, whereas the proportion of predators attached to the coaggregates further increased, representing half of the population at the end of the experiment (Fig. 4). The feeding activity of this large population of predators likely removed all the newly produced bacterial cells (resulting in stable bacterial total numbers). In turn, it probably provided the coaggregates with a local growth-stimulating source of organic and inorganic components, supporting the productivity of the microbial hot spot (Hagström et al. 1988). Flagellates are known to excrete about 30-40% of the ingested C as labile DOC (Chase and Price 1997; Pelegrí et al. 1999). The emerging interaction between the two

competing prey species and the predators thus resulted in an extremely productive microenvironment, characterized by an inverted trophic pyramid where a limited amount of prey could sustain a larger population of specialized predators (Wiggert et al. 2005; Miyazaki et al. 2006). Particle-bound flagellates in the pelagic zone of lakes can be efficient grazers of planktonic bacteria (Simek et al. 1997). Bacterial biofilms and planktonic aggregates in nature are often densely colonized by a variety of protozoa that directly impact on bacterial dynamics on particle surfaces (Arndt et al. 2003, Wey et al. 2008) and that possibly also control their abundances (Kiørboe et al. 2004). In fact, the attachment to surfaces is regarded as the most favorable strategy for suspension-feeding protozoans, promoting higher bacterial uptake rates and allowing for higher growth rates (Christensen-Dalsgaard and Fenchel 2003). In our system, such a surface for the attachment of the predators was provided by bacterial coaggregates. Since these coaggregates were largely composed of edible *Brevundimonas* sp. cells, it is conceivable that a large number of these bacteria were also released into the surrounding medium, where they were consumed by the attached flagellates.

In some aspects, the colonized coaggregates in our experiments may represent simplified models for the bacterial assemblages on organic aggregates that are common in the pelagic zones of oceans (marine snow, Alldredge and Silver 1988) and freshwaters (lake snow, Grossart and Simon 1993). The general concept of bacteria able to colonize larger floating particles (Simon et al. 2002) might be extended to their attachment to planktonic bacterial aggregates or microcolonies, thus flocculating by themselves, as seen in highly productive environments (Malik et al. 2003). Bacteria on such flocks may consume a considerable fraction of total primary production, and are responsible for a large proportion of the flux of DOC within pelagic food webs (Hoppe et al. 2002, Azam and Malfatti 2007). In addition, the proximity of microorganisms within a flock promotes their chance of interaction, e.g. via cell-to-cell signaling (Gram et al. 2002). Finally, there are indications that bacterivorous flagellates may accumulate in the vicinity of such natural organic aggregates or even

attach to them (Kiørboe et al. 2002), resulting in a system where a large population of predators is maintained by a smaller, but extremely active, population of prey (Hutchinson 1961).

Our results call for a reinterpretation of the basic concept of bacteria as being links or sinks (Sherr et al. 1987) for DOC, taking also into account the spatial organization of bacterial assemblages and the interactions between their individual members. In addition, they also highlight the importance of hidden ecological interactions and emerging properties for understanding the functioning of even simple microbial communities. We show that such interactions can be readily revealed in clean experimental setups by a choice of model organisms that is guided by a priori ecological reasoning, i.e., by selecting bacterial strains that also co-occur and interact in the natural habitat. It is intriguing to speculate that the enhanced transfer of DOC to the higher trophic level observed in our simple experimental food web (Fig. 6) might be a more general principle, i.e., that coaggregation and emerging interactions between attached bacteria might positively affect the overall efficiency of the microbial loop in aquatic habitats.

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FIGURE LEGENDS

FIG. 1: Bacterial abundances in pure and cocultures, with and without predators . Total bacterial numbers (first row), numbers of cells in microcolonies < 10 μm (second row) and in aggregates (third row). Error bars: SD of 3 replicates.

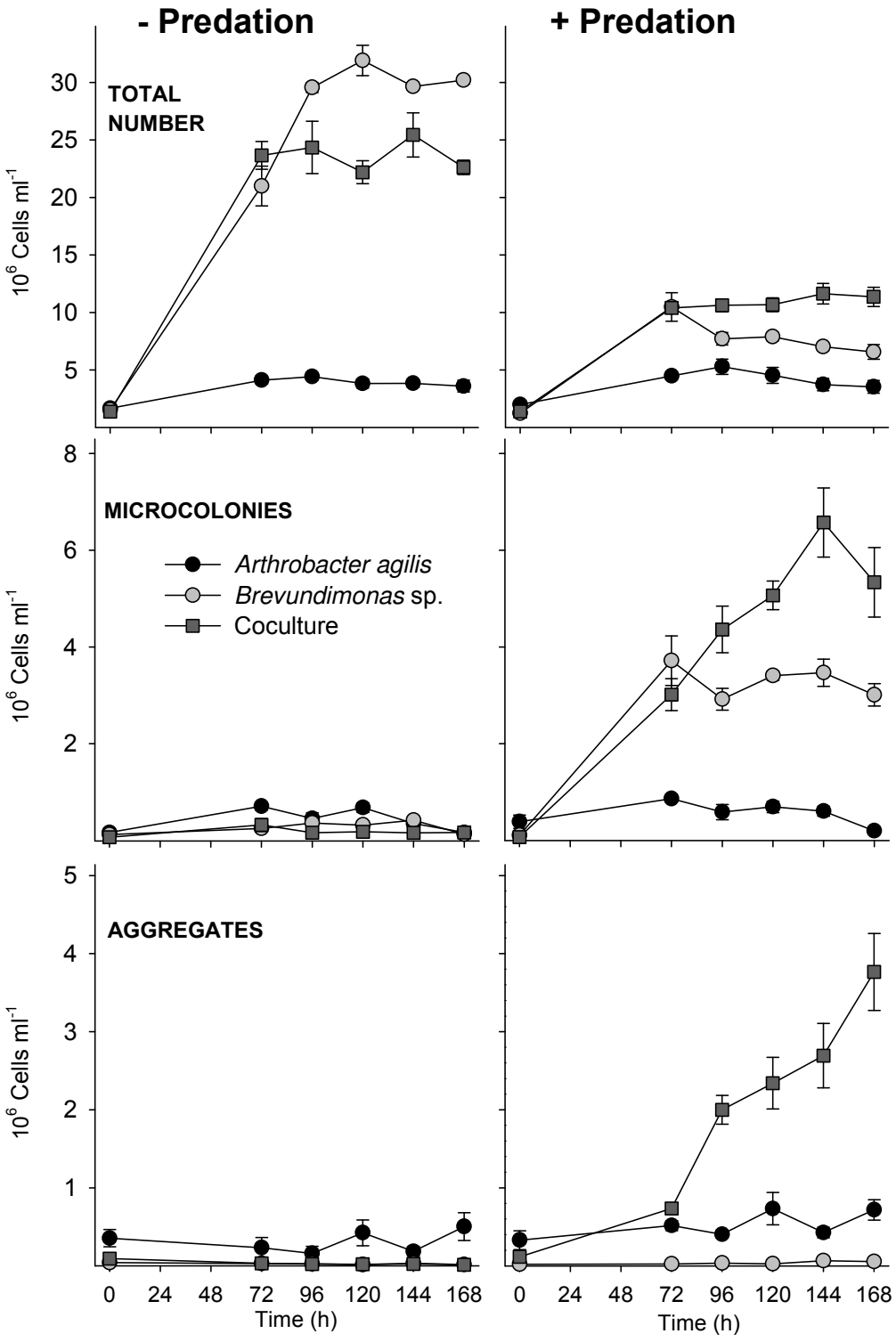
FIG. 2: A) Proportion of the different growth types (single cells, microcolonies and aggregates; stacked bars). B) Relative size distribution of aggregates and coaggregates in cocultures after 96h to 168h of growth. Insets: Photomicrograph of bacterial coaggregates (i) depicting *Brevundimonas* sp. cells in blue (DAPI staining) and *A. agilis* in pink (CARD-FISH staining); photograph of a co-colony (ii) of the two strains (*A. agilis*, red, in the centre and *Brevundimonas* sp., orange, peripheral) on agar plate after flow cytometric sorting of coaggregates.

FIG. 3: Total numbers of formed cells within the different growth types (single cells, MC = microcolonies, AGGR = aggregates) measured per strain, in single and cocultures (means of the stationary growth period between 96 and 168h, SD on the overall bacterial abundances). Upper stacked bars refer to *A. agilis*, and lower ones to *Brevundimonas* sp.. The inset in upper graph shows the respective trends in the relative proportions of *A. agilis* in coculture during the same period with (+Pred) and without (-Pred) predators.

FIG. 4: Lines and symbols: Development of total numbers of *Poterioochromonas sp.* strain DS (predators) in single cultures of *A. agilis* (A), of *Brevundimonas* sp. (B), and in cocultures (C) (means ±SD of 3 replicates). Pie charts: relative proportion of free living and aggregate-attached cells of *Poterioochromonas sp.* strain DS in the same treatments (means all sampling points within the stationary growth period between 96 and 168h).

FIG. 5: Proportion of aggregates/coaggregates colonized by *Poterioochromonas sp.* strain DS in cocultures during the stationary growth phase between 96h and 168h. Light and dark grey areas refer to aggregates composed of (and coaggregates dominated by) *Brevundimonas* sp. or *A. agilis,* respectively. Right panel: photomicrograph of a bacterial coaggregate colonized by predators (small bacterial cells in blue: *Brevundimonas* sp.; large cells in light blue: *Poterioochromonas* sp. strain DS [DAPI staining]; pink cells: *A. agilis* [CARD-FISH-staining]).

FIG. 6: (A) Numbers of edible (single) bacterial cells available per predator during the stationary growth period between 96h and 168h; the broken line depicts the theoretical threshold of 1000 bacterial cells per flagellate cell evidences that distinguishes between a system mainly controlled by bacterial competition (>1000), or by predation (<1000). B): Estimated proportions of particulate organic carbon bound in bacteria and flagellates during the same period; the broken line indicates the amount of dissolved organic carbon available in the cultures at the beginning of the experiment. The white bars indicated the estimated C in bacterial biomass necessary to sustain the flagellates community of each treatment, as proposed by Fenchel (1982).


- Predation
+ Predation
TOTAL NUMBER
MICROCOLONIES
AGGREGATES
Arthrobacter agilis
Brevundimonas sp.
Coculture
10^6 Cells ml^-1
Time (h)
0 24 48 72 96 120 144 168

1

2 FIG 1

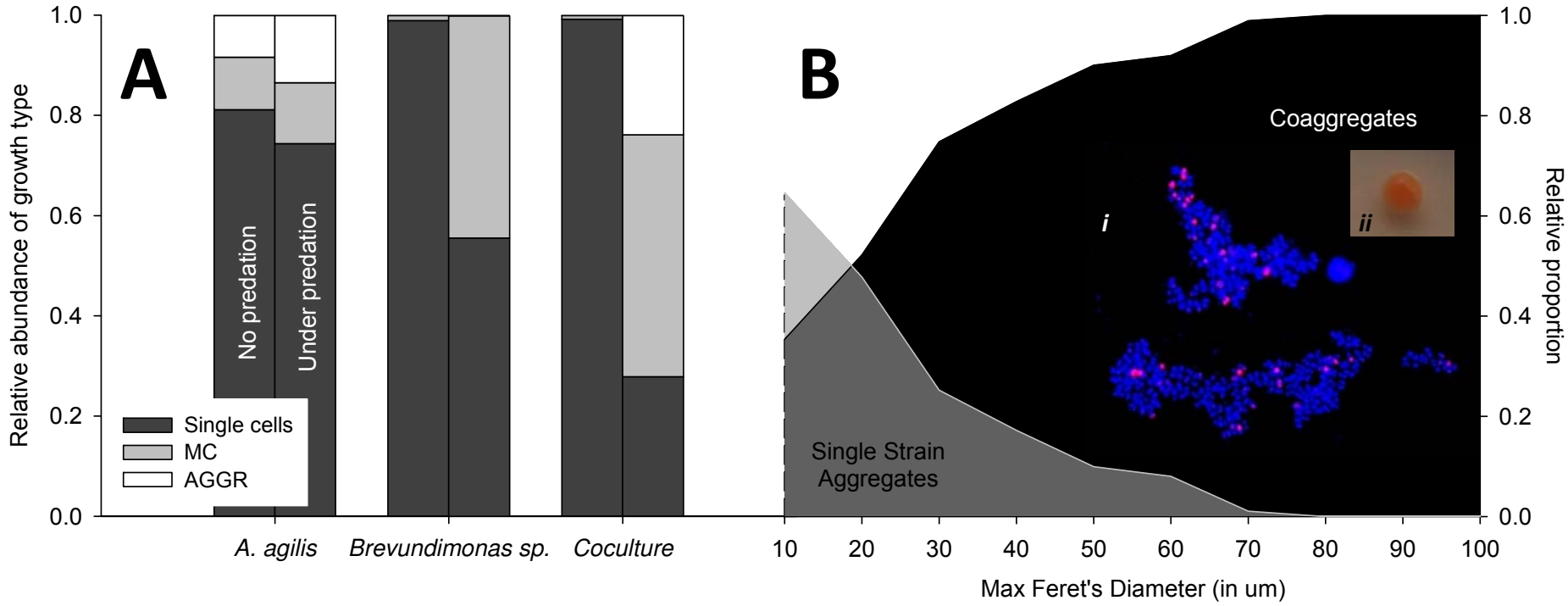

A
B
Relative abundance of growth type
1.0
0.8
0.6
0.4
0.2
0.0
No predation
Under predation
Single cells
MC
AGGR
A. agilis
Brevundimonas sp.
Coculture
Single Strain
Aggregates
Coaggregates
i
ii
10
20
30
40
50
60
70
80
90
100
Max Feret's Diameter (in um)
Relative proportion

FIG. 2

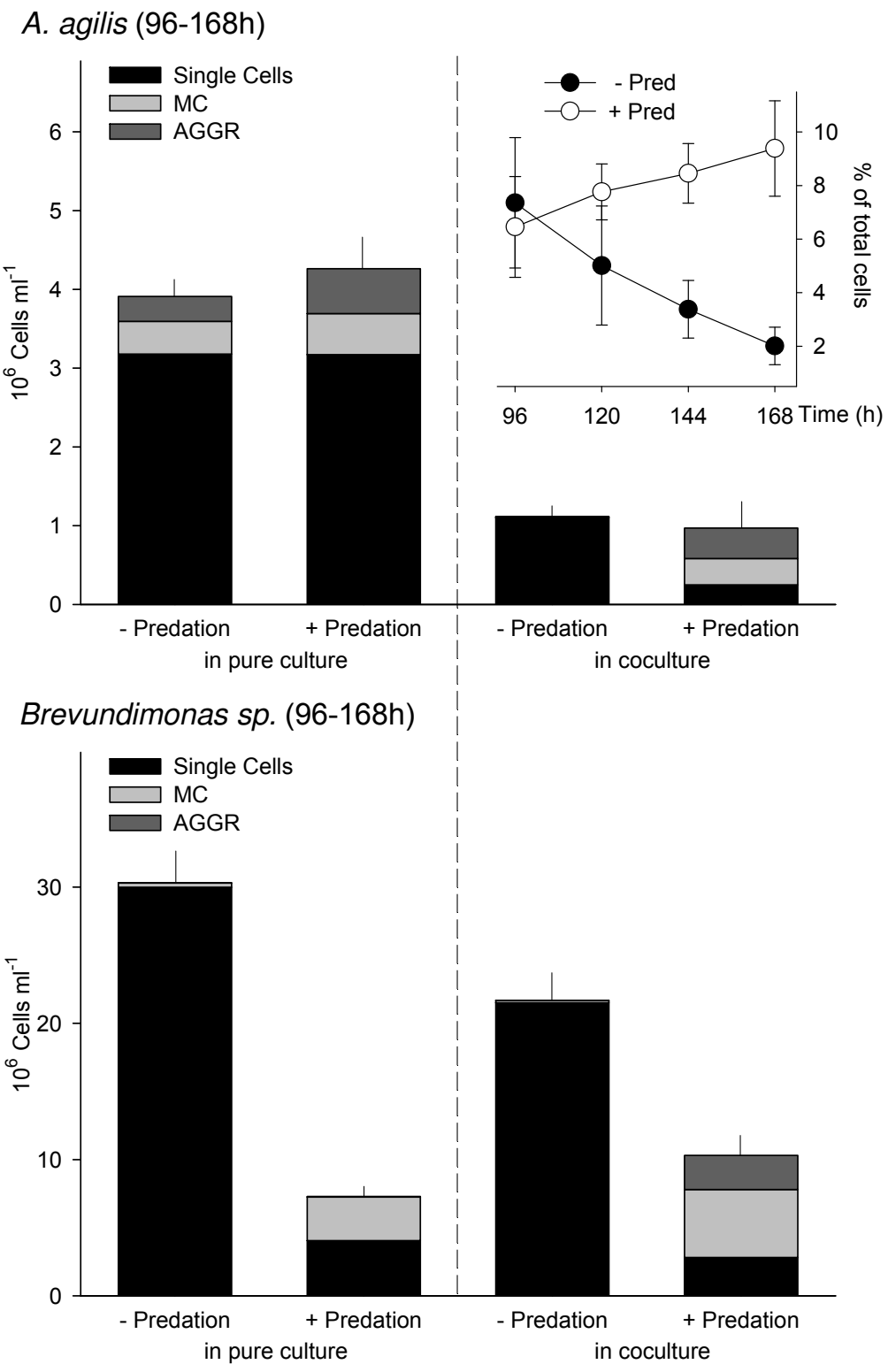
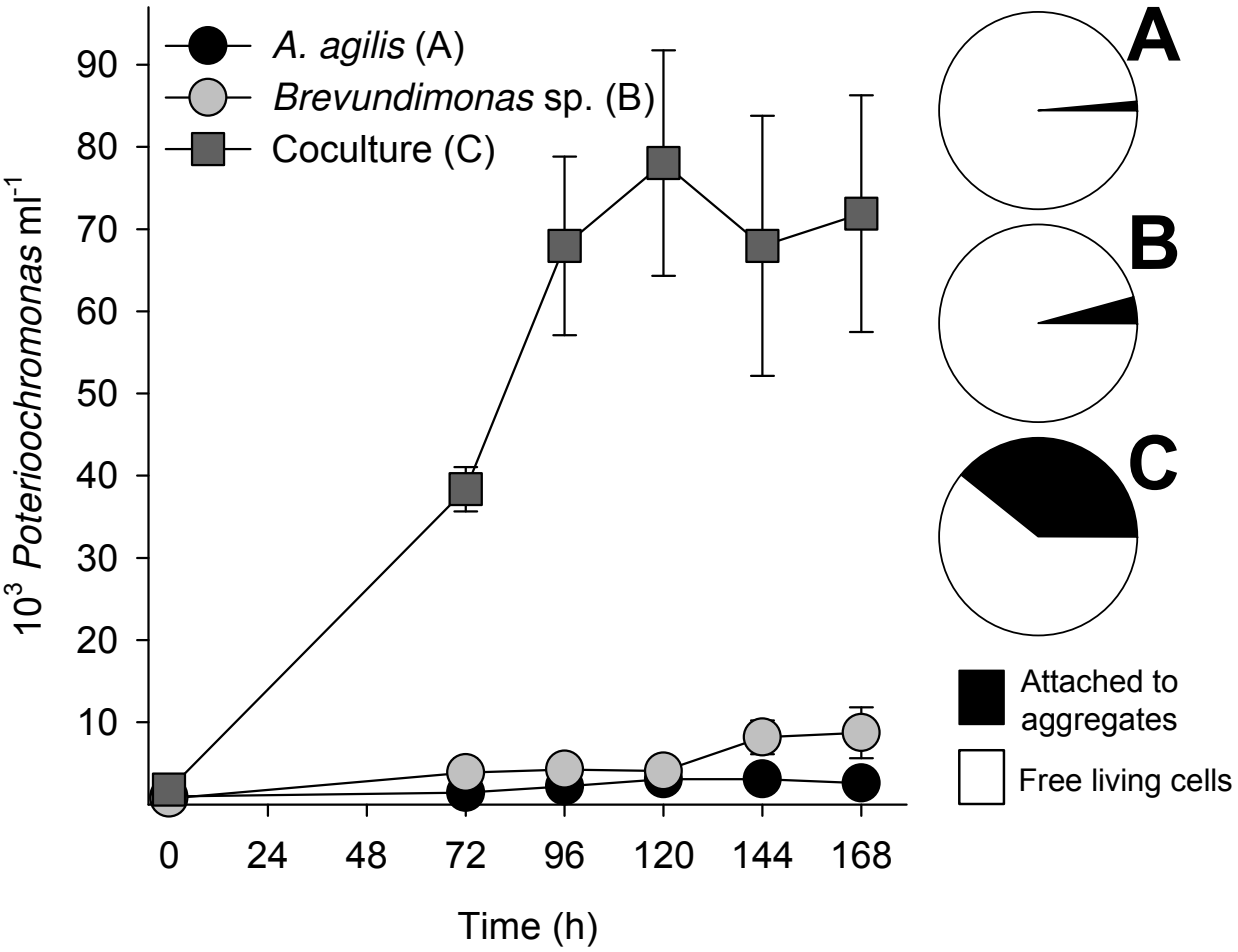

A. agilis (96-168h)
Single Cells
MC
AGGR
- Pred
+ Pred
10^6 Cells ml^-1
% of total cells
Time (h)
96
120
144
168
- Predation
+ Predation
in pure culture
in coculture
Brevundimonas sp. (96-168h)

FIG. 3


A. agilis (A)
Brevundimonas sp. (B)
Coculture (C)
10^3 Poterioochromonas ml^-1
90
80
70
60
50
40
30
20
10
0
24
48
72
96
120
144
168
Time (h)
A
B
C
Attached to aggregates
Free living cells

1

2 FIG. 4

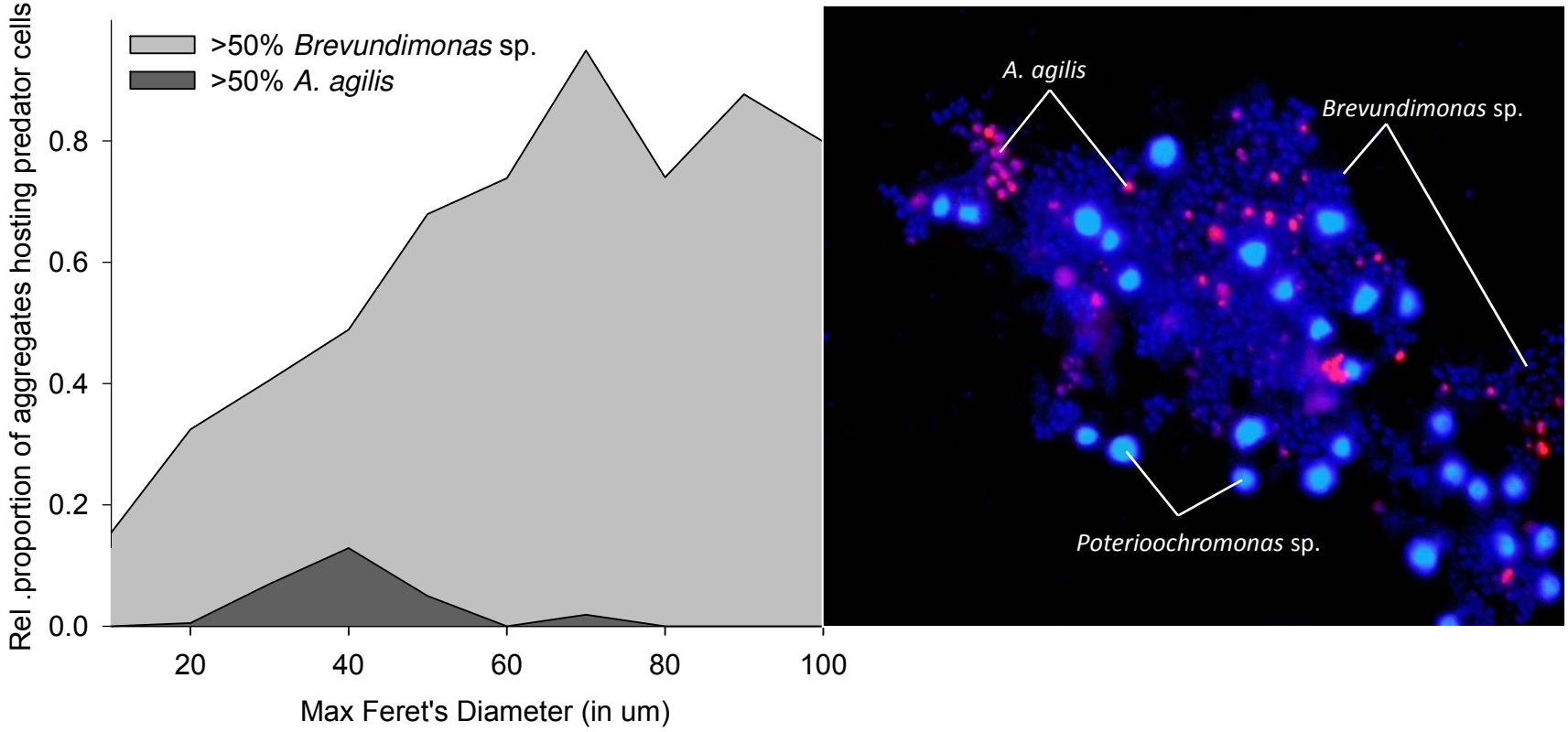
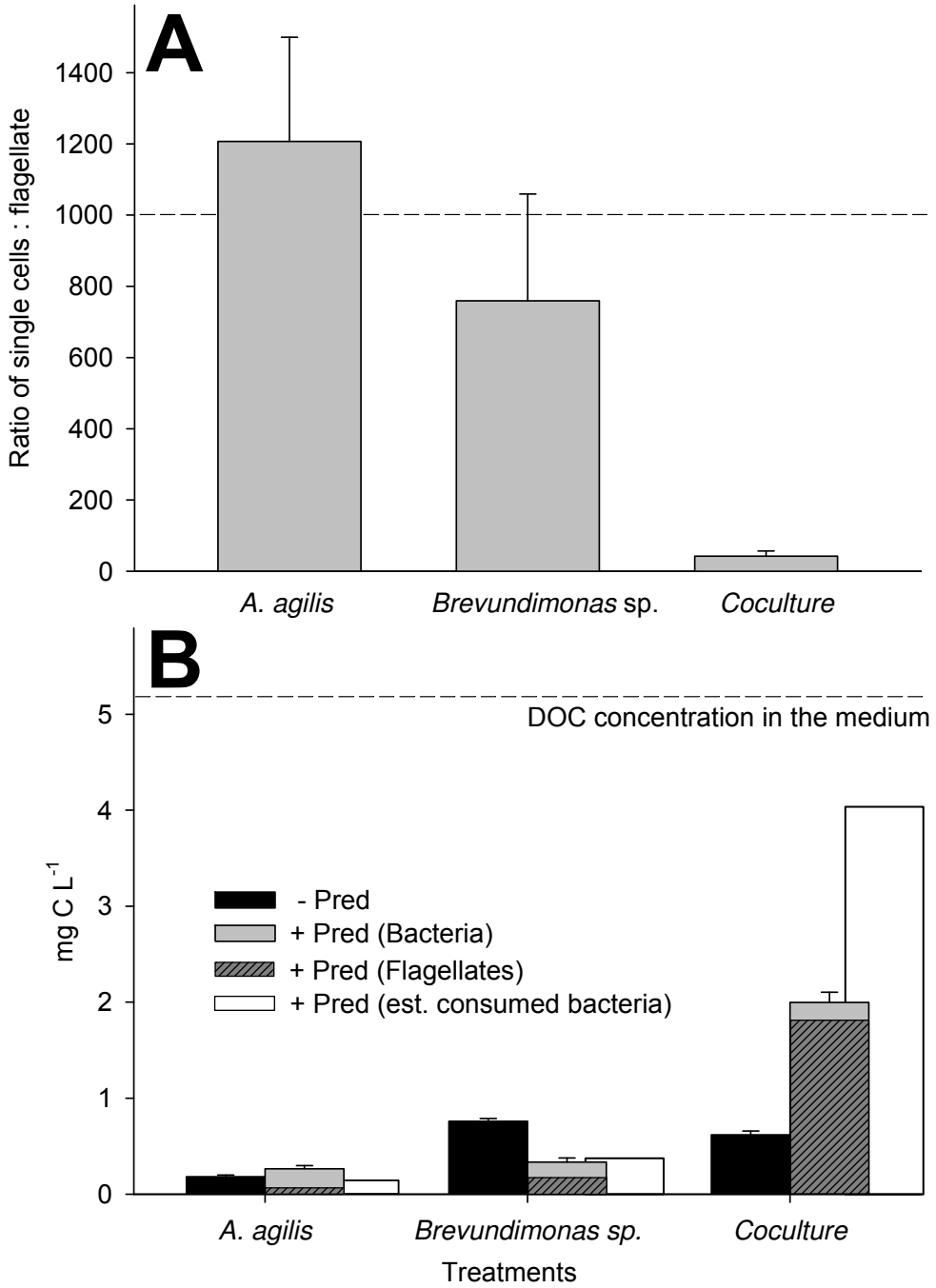

>50% Brevundimonas sp.
>50% A. agilis
Rel .proportion of aggregates hosting predator cells
0.0
0.2
0.4
0.6
0.8
20
40
60
80
100
Max Feret's Diameter (in um)
A. agilis
Brevundimonas sp.
Poterioochromonas sp.

FIG. 5


A
Ratio of single cells : flagellate
1400
1200
1000
800
600
400
200
0
A. agilis
Brevundimonas sp.
Coculture
B
DOC concentration in the medium
mg C L-1
5
4
3
2
1
0
- Pred
+ Pred (Bacteria)
+ Pred (Flagellates)
+ Pred (est. consumed bacteria)
A. agilis
Brevundimonas sp.
Coculture
Treatments

1

2 FIG. 6

Treatment		Cocult *vs* Pure Cult		Same Treatment	Trend *vs* Time			
					Pure culture		Coculture	
		No Pred	Pred	Pred *vs* NoPr	No Pred	Pred	No Pred	Pred
Brevundimonas sp.	Total	●●●(P)	●(C)	●●●(NP)	=	=	●↑	=
	Single Cells	●(P)	=	●●●(NP)	=	●↓	=	●↓
	Microcolonies	=	●(C)	●●●(Pr)	=	=	=	●●●↑
	Aggregates	-*	●●●(C)	-*	-*	-*	-*	●●●↑
Arthrobacter agilis	Total	●●●(P)	●●●(P)	=	=	=	●●↓	●↑
	Single Cells	●●●(P)	●●●(P)	=	=	=	●●↓	●●↓
	Microcolonies	●●●(P)	=	=	=	=	●↓	=
	Aggregates	●●(P)	=	=	=	=	=	●●↑
Cocultures (overall)	Total	-	-	●●(NP)	-	-	=	=
	Single Cells	-	-	●●(NP)	-	-	=	●●↓
	Microcolonies	-	-	●●●(Pr)	-	-	=	●●↑
	Aggregates	-	-	●●●(Pr)	-	-	=	●●●↑

TABLE 1. Comparison of overall abundances and growth type distribution during the stationary growth phase (96-168h) between Coculture (C) and Pure culture treatments (P) without (first column) and with predators (second column), and between the same treatments (pure cultures for each strains and coculture) with (Pr) and without (NP) predators (third column). Stationary growth phase trends for overall and growth type abundances in the different treatments. Significance: ●●● for $P<0.001$; ●● for $P<0.01$; ● for $P<0.5$; = for non-significant differences and horizontal trends; - for analysis non performed. ↑ indicates a trend towards higher abundances while ↓ indicates a trend towards decrement. * refers to statistical analyses involving comparisons of *Brevundimonas* sp. aggregate numbers in Pure and Co-cultures, too low to be considered.